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Our goal is to understand the process of DNA replication at the molecular level in human cells. We are specifically trying to identify human replicator sequences and to characterize the pre-replicative complexes at the origins of replication. In this report we summarize a series of experiments using chromatin-immunoprecipitation techniques and discuss the technical problems associated with them. Then we evaluate the stability of the human origin recognition complex across the cell cycle and present a novel regulation of its large subunit, hOrc1p, that is very relevant to our main research interests.

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INTRODUCTION

The process of cell proliferation is the essence of life. Each time a human cell divides, the genomic information contained in its chromosomes is first duplicated and then distributed between the two daughter cells. Normal cells display multiple mechanisms to ensure that the transmission of genetic information is accurate. Occasionally, some cells accumulate genetic lesions (mutations, deletions, reorganizations of the chromosomes) that abolish the proliferation controls and lead to the onset of cancer. Our laboratory studies the function of several human genes, as the Origin Recognition Complex (ORC), Cdc6 and Mini-Chromosome Maintenance (MCM) that participate in the process of DNA replication. Previous work in model systems such as yeast, *Xenopus* and *Drosophila* have shown that these genes are important for the accurate replication of the genome. At least in yeast and *Drosophila*, ORC proteins recognize and bind to specific "replicator" DNA sequences, located in the vicinity of the actual origins of replication. ORC serves as the core for the formation of a multi-protein structure called **pre-replicative complex** that includes Cdc6, MCM proteins and possibly other factors. Pre-replicative complexes render the origins of replication competent to initiate the duplication process, and should be regulated to ensure that each DNA molecule is replicated once and only once before cell division. The main goals of this project are the characterization of human pre-replicative complexes and the identification of human replicator sequences. This will allow a better understanding of the molecular events that lead to DNA replication and their potential connection to cellular transformation.

BODY

Our initial experiments to characterize human origins of replication used the Chromatin Immuno-Precipitation (ChIP) assay. In this method, cellular proteins associated with the chromatin are crosslinked *in vivo* to the DNA and the resultant chromatin is fragmented in small molecules, that are incubated with specific antibodies to the protein of interest. The co-precipitated DNA sequences are then identified using PCR amplification reactions. As it was indicated in the first Annual Report (August 2000), an experimental protocol was optimized for this purpose, and its validity was tested with a transcription factor whose binding site in human DNA was already characterized. We subsequently applied this protocol to try to identify DNA binding sequences for the human Origin Recognition Complex (hORC). Given the high complexity of the human genome, we focused in a specific region that contains an active origin of replication. The β-globin gene locus origin of replication has been identified using diverse physical methods[1], but whether it contains binding sites for human ORC remained to be tested.

The ChIP experiments with hORC have proven to be technically difficult and the results obtained were not conclusive. After chromatin crosslinking, immunoprecipitation with a monoclonal anti-hOrc2 antibody and PCR analysis with specific primer pairs across the β-globin region, in some experiments we detected a preferential representation of a particular region within the locus (nucleotides 62,202-62,597), but this enrichment was within the margin of assay-to-assay variability.

During the last year, we have evaluated the different technical steps in our protocol that could influence the experimental outcome, and we have introduced and tested a number of modifications in the original method. The sensitivity of the assay was improved by increasing the starting concentration of chromatin up to 10-fold, but similar results were obtained. We considered the possibility of epitope masking -i.e. the hOrc2p epitope recognized by the monoclonal antibody could be hidden by other components of the pre-replicative complex. To overcome this potential problem, ChIP experiments were performed with different antibodies raised in the laboratory against other components of the pre-replicative complex, such as Orc6, Cdc6 and MCM proteins[2]. Intriguingly, in all cases we obtained similar results –all regions within the β-globin locus seemed to be

equally represented. Although these results could argue as against the existence of specific hORC DNA binding sites, it is not possible to draw this conclusion from a negative result.

We then considered the possibility that human ORC might be unstable in human cells, as this could explain the difficulty in identifying its binding sites. The relative abundance of different subunits of hORC in different stages of the cell cycle was measured in HeLa cells isolated by centrifugal elutriation (see Appendix, Figure 1). The different hORC subunits were detected by immunoblotting with specific, affinity-purified antibodies. hOrc1p accumulated during G1, but its levels decreased significantly after the G1-S transition and were very low during the S and G2 phases. The degradation of hOrc1p was concomitant with the expression of cyclin A. In contrast, hOrc2p, hOrc3p or hOrc6p subunits were present at constant levels across the cell cycle. This result suggests that hOrc1p is a labile subunit within the hORC and it might be a limiting factor for initiation of DNA replication. It should be noted that this regulation of hOrc1 is different from yeast, in which all ORC subunits are associated with the origins of replication during the whole cell cycle[3].

We have studied the molecular events that could lead to the degradation of hOrc1p after the cells have entered S phase. The protein levels of the most important cell cycle regulators, such as cyclins and cyclin-CDK inhibitors, are controlled by ubiquitin-mediated proteolysis[4]. The Skp1-cullin-F-box protein complex (SCF) and the anaphase promoting complex (APC) are the two major cellular ubiquitin-conjugating complexes that regulate cell cycle progression. The SCF regulates the proteolytic events that drive the cells through the G1-S transition. It is composed of Skp1, Cul1 and Rbx1, as well as a variable component called the F-box protein that provides substrate specificity[4]. Skp2 is an F-box protein that was originally identified as a cyclin A-CDK interacting protein[5]. Considering the fluctuation of hOrc1p in the cell-cycle (Appendix, Figure 1) and the fact that hOrc1p interacts with cyclin A-CDK2[6], we tested whether Skp2 could also interact with hOrc1p and therefore participate in its polyubiquitination. In collaboration with graduate student X. Helena Zou-Yang, we found that recombinant GST-Skp2 protein, expressed and purified from *E. coli*, pulled-down *in vitro* translated hOrc1p but not *in vitro* translated hOrc2p. This result was confirmed by immunoprecipitation from whole

cell extracts expressing T7-hOrc1p. Anti-Skp2 antibodies, but not a control pre-immune serum, coimmunoprecipitated a fraction of the T7-hOrc1p protein present in the extract. These results suggest that Skp2 might participate in hOrc1p ubiquitination.

The involvement of the polyubiquitination machinery in the turnover of hOrc1p was confirmed with an *in vivo* ubiquitination assay that has been useful to detect polyubiquitinated intermediates of other proteins such as c-Jun[7], c-Myc[8] and hCdc6p[2]. HeLa cells were co-transfected with a plasmid expressing T7-tagged hOrc1p and a plasmid expressing $(His)_6$-tagged ubiquitin. Cell extracts were prepared 36 h post-transfection and the ubiquitinated proteins were purified by affinity chromatography. Only when cells expressed both T7-hOrc1p and His-Ubi, a smear of purified products of high MW were detected with anti-T7 antibodies, likely corresponding to multi-ubiquitinated hOrc1p intermediates. None of these products were detected in the absence of His-Ubi (see Appendix, Figure 2A). To test if the 26S proteasome was involved in the regulated destruction of hOrc1p, HeLa cells were treated with different proteasome inhibitors. The steady-state levels of hOrc1p were significantly increased when HeLa cells were treated with MG132 and β-lactone, whereas the levels of hOrc2p were not affected (Appendix, Figure 2B).

Finally, we have also found that hOrc1p is limiting under a situation of cellular stress. HeLa cells were treated with the chemotherapeutic agent adriamycin, which produces double stranded DNA breaks and eventually leads to p53-induced apoptosis. The treatment with adriamycin greatly reduced the amount of chromatin-associated hOrc1p, whereas hOrc2p was not affected. The degradation of hOrc1p in a situation of DNA damage is likely to prevent further initiation events.

These observations have helped us to understand the difficulty of finding human ORC DNA binding sites, as the largest subunit of the human ORC seems to be unstable during a large part of the cell cycle. On the other hand, the novel findings are encouraging in the sense that the observed association of hOrc1p with chromatin during G1, followed by its degradation at S phase, correlates remarkably well with the *in vivo* footprint analysis at the lamin B2 human origin of replication[9]. No footprinting is observed during mitosis, but a region spanning 100 bp is protected during G1, that shrinks to 70 bp after the cells enter S phase[9]. One of the next critical questions will be to

determine whether hOrc1p protein is responsible for these protein-DNA interactions at the lamin B2 region, or other potential human origins of replication.

Experiments are underway that include the synchronization of cells in G1 phase (either by centrifugal elutriation or mitotic block and release) as a preliminary step before the ChIP assay described above. The cells isolated in G1 should have an intact hORC complex and this might be essential for the success of the project.

KEY RESEARCH ACCOMPLISHMENTS

1. We have evaluated the stability of the human origin recognition complex across the cell cycle. The levels of hOrc1p are regulated in the cell cycle, being abundant in G1 and lower after the cells enter S phase. In contrast, other hORC subunits are stable.

2. hOrc1p interacts with F-box protein Skp2, suggesting the involvement of the SCF ubiquitin-conjugating machinery and the 26S proteasome in the degradation of hOrc1p.

3. hOrc1p can be polyubiquitinated *in vivo*, and its steady-state levels are increased by inhibition of the 26S proteasome.

4. hOrc1p is degraded when cells are treated with the DNA-damaging agent adriamycin, likely to prevent further initiation events.

5. This new information is currently being used to design improved chromatin immunoprecipitation experiments to identify human replicator sequences.

REPORTABLE OUTCOMES

1. Abstract submitted to "The cell cycle Meeting". Salk Institute, 2001:

HUMAN ORIGIN RECOGNITION COMPLEX LARGEST SUBUNIT IS DEGRADED BY UBIQUITIN-MEDIATED PROTEOLYSIS AFTER INITIATION OF DNA REPLICATION

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Eukaryotic cells display overlapping mechanisms to ensure that DNA replication is restricted to the S phase of the cell cycle. We find that the levels of hOrc1p, the largest subunit of the human origin recognition complex, are tightly controlled in the cell cycle. During mitosis hOrc1p chromatin association is prevented. The protein is expressed and targeted to chromatin in early G1, when pre-replicative complexes are formed at origins of DNA replication. In contrast, hOrc2p is stable throughout the cell cycle, and 50% remains bound to chromatin during mitosis. Unlike yeast Orc1p, hOrc1p levels decrease abruptly as cells enter S phase and cyclin A accumulates. Cyclin A-CDK2 interacted with hORC and phosphorylated hOrc1p and hOrc2p. HOrc1p levels were controlled by its polyubiquitination and destruction by the 26S proteasome. hOrc1p interacted with the F-box protein Skp2, suggesting the involvement of the Skp1-Cullin-F-box protein (SCF) ubiquitin conjugating machinery. Specific degradation of hOrc1p was also observed upon induced DNA damage. This novel regulation of hOrc1p can contribute to the maintenance of ploidy in human cells.

2. Manuscript submitted for publication (Molecular Cell, June 2001):

J. Méndez, X. Helena Zou-Yang, M. Hidaka and B. Stillman. 2001. Human origin recognition complex largest subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication

CONCLUSIONS

The identification of human replicator sequences using chromatin immunoprecipitation (ChIP) assays in asynchronously growing cells has been difficult. We have investigated the factors that could explain this difficulty and found that the large subunit of the human Origin Recognition Complex is unstable in the cell cycle. HOrc1p is polyubiquitinated and degraded by the proteasome as the cells progress through S phase. This could result in the dissociation of part of the ORC complexes from chromatin. HOrc1p interacts with F-box protein Skp2, suggesting the involvement of the SCF ubiquitin-ligase machinery in the control of hOrc1p levels. The link between hOrc1p and Skp2 is potentially very interesting. Skp2 is oncogenic and overexpressed in human cancers[10]. Interestingly, targeted disruption of Skp2 in mice results in high levels of $p27^{Kip1}$, cyclin E and polyploidy in some cell types[11]. Hepatocytes from the Skp2-/- animals can accumulate up to 16N DNA. This could be explained by $p27^{Kip1}$-mediated inhibition of kinase Cdc2, resulting in cells that cannot progress through mitosis. As we find that hOrc1p levels are controlled by the SCF, likely in a Skp2-dependent manner, it is possible that the inability to degrade hOrc1p could be one of the factors leading to genome overreplication.

All the results collected during the last year and presented in this report emphasize the importance of using a population of cells isolated in the G1 phase of the cell cycle as starting material for the chromatin immunoprecipitation experiments. The integrity of the human ORC is likely essential in order to identify its DNA binding sites. Experiments are underway to address this issue.

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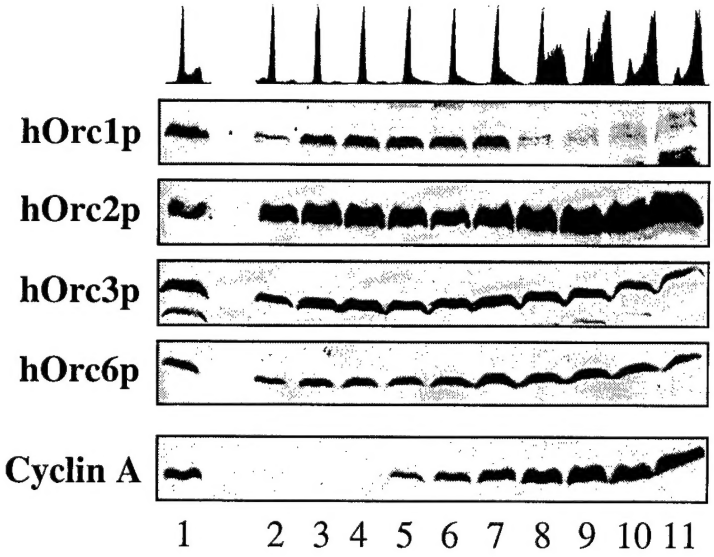

FIGURE 1. hOrc1p is regulated during the cell cycle. HeLa cells were separated in different stages of the cell cycle by centrifugal elutriation. The DNA content for the cells in each fraction is shown. Equivalent amounts of total cell extracts (normalized by cell number) were subjected to SDS-PAGE and analyzed by immunobloting with the indicated antibodies.

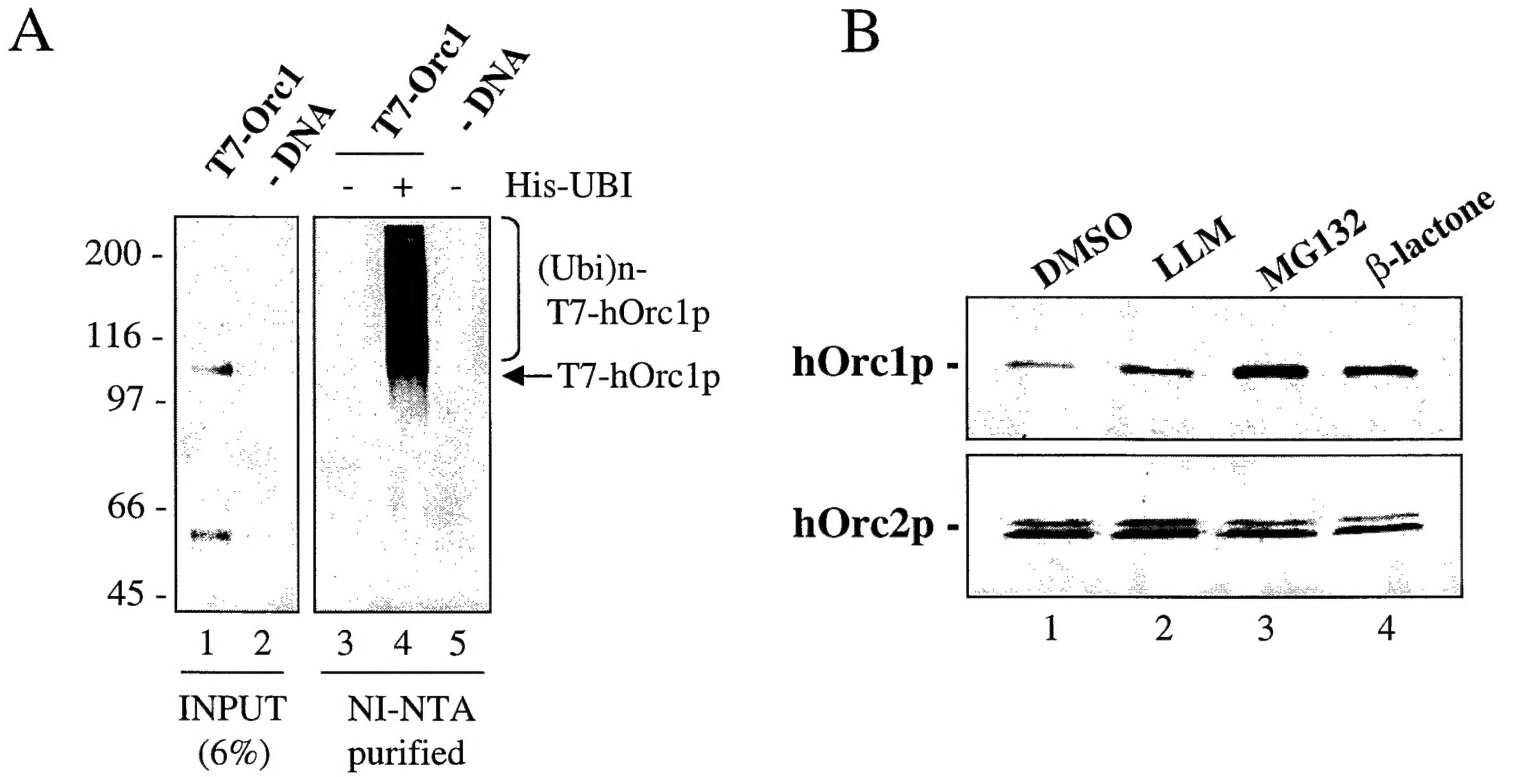

FIGURE 2. A. hOrc1p can be polyubiquitinated *in vivo*. HeLa cells were transiently transfected with a plasmid expressing T7-hOrc1p in the absence or in the presence of pMT107 (expressing His-tagged ubiquitin). Transfected cells were harvested, lysed and the ubiquitinated proteins were purified in a Ni-NTA affinity column. Purified proteins were subjected to SDS-PAGE and analyzed by immunobloting with anti-T7 antibody. Polyubiquitinated proteins are tipically detected as a smear of high-MW products. **B. Proteasome inhibition**. HeLa cells were incubated for 6 h in regular medium supplemented with LLM, MG132 or β-lactone, Control cells were treated with equivalent amounts of dimethyl sulfoxide (DMSO). Total cell extracts were prepared and the abundance of hOrc1p and hOrc2p was analyzed by immunoblotting with specific antibodies.